THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 266, No. 11, Issue of April 15, pp. 6909-6914, 1991
Printed in U.S.A.

Expression of a Whey Acidic Protein Transgene during Mammary Development

EVIDENCE FOR DIFFERENT MECHANISMS OF REGULATION DURING PREGNANCY AND LACTATION*

(Received for publication, September 27, 1990)

Tom Burdon‡§, Lakshmanan Sankaran¶, Robert J. Wall‖, Mark Spencer‖, and Lothar Hennighausen‡**

From the ‡Laboratory of Biochemistry and Metabolism and the ¶Diabetes Branch, National Institue of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892 and the ‖U. S. Department of Agriculture, Agricultural Research Service, Beltsville, Maryland 20725

Expression of the mouse whey acidic protein (WAP) gene is specific to the mammary gland, is induced several thousand-fold during pregnancy, and is under the control of steroid and peptide hormones. To study developmental regulation of the mouse WAP gene, a 7.2-kilobase (kb) WAP transgene, including 2.6 kb of 5′- and 1.6 kb of 3′-flanking sequences, was introduced into mice. Of the 13 lines of mice examined, 6 expressed the transgenes during lactation at levels between 3 and 54% of the endogenous gene. Although expression was dependent on the site of integration, the transgenes within a given locus were expressed in a copy number-dependent manner and were coordinately regulated. The WAP transgenes were expressed specifically in the mammary gland, but showed a deregulated pattern of expression during mammary development. In all six lines of mice, induction of the WAP transgenes during pregnancy preceded that of the endogenous gene. During lactation, expression in two lines increased coordinately with the endogenous gene, and in three other lines of mice, transgene expression decreased to a basal level. These data indicate that the 7.2-kb gene contains some but not all of the elements necessary for correct developmental regulation. At a functional level it appears as if a repressor element, which inactivates the endogenous gene until late pregnancy, and an element necessary for induction during lactation are absent from the transgene. Complementary results from developmental and hormone induction studies suggest that WAP gene expression during pregnancy and lactation is mediated by different mechanisms.

Expression of milk protein genes is dependent upon interactions between tissue-specific and developmentally and hormonally induced regulatory factors (1). Our laboratory uses the mouse whey acidic protein (WAP)[1] gene as a model to study the structure and function of the corresponding regulatory elements. The WAP gene encodes the major whey protein in mice (2), rats (1), and rabbits (3) and is expressed almost exclusively in the mammary gland. The steady state level of mouse WAP mRNA increases several thousand-fold between the virgin state and mid-lactation (4, 5). This induction depends upon the presence of lactogenic hormones, glucocorticoids, and insulin (5) but may also require some, as yet undefined, features of cell-cell interactions occurring within the mammary gland (6).

WAP gene expression increases sharply between day 15 and 17 of pregnancy (5), a period during which the levels of placental lactogens are near maximal but prolactin levels are low (7). Insulin and hydrocortisone are present throughout pregnancy. In contrast to WAP the β-casein gene is induced at day 10 of pregnancy (8), coincident with the increase in placental lactogens (7). In spite of their different temporal patterns of expression during pregnancy, induction of both the WAP and β-casein genes in organ explant cultures from mid-pregnant mice requires all three hormones, insulin, hydrocortisone, and prolactin (5, 8–10). The mechanisms by which these hormones activate milk protein gene expression is unclear. Doppler *et al.* (11) have shown in tissue culture cells transfected with a β-casein gene that prolactin and hydrocortisone act through promoter sequences and therefore presumably on the transcriptional level. However, Rosen and co-workers (12) have evidence that these hormones act predominantly at a post-transcriptional level.

Previous studies have shown that a hybrid gene containing 5′- flanking sequences of the mouse WAP gene is expressed specifically in the mammary gland of transgenic mice (4, 13), suggesting that mammary-specific elements reside in 2.6 kb of the promoter upstream region. However, correct developmental and hormonal regulation of the chimeric genes was not observed (5). To test whether sequences downstream from the promoter are required for correct regulation of the WAP gene, we have introduced into mice a 7.2-kb fragment of DNA which encompasses the entire transcribed region of the mouse WAP gene, 2.6 kb of 5′- and 1.6 kb of 3′-flanking DNA. Analysis of WAP transgene expression in six independent lines of mice allowed us to study the contribution of regulatory elements within the 7.2 kb to the developmental and hormonal regulation of the mouse WAP gene. In addition, the introduction of three different WAP gene alleles into a single integration site allowed us to evaluate the influence of surrounding chromatin on the level of expression and developmental regulation of individual transgenes within one locus.

MATERIALS AND METHODS

Recombinant Plasmids—The plasmid pBS WAP, containing a 7.2-kb *Eco*RI fragment with the mouse WAP gene (14), was linearized

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a postdoctoral fellowship from the Cystic Fibrosis Foundation.

** To whom correspondence should be addressed. Tel.: 301-496-2716; Fax: 301-496-0839.

[1] The abbreviations used are: WAP, mouse whey acidic protein; kb, kilobase(s); PCR, polymerase chain reaction.

with either *Kpn*I (exon 1), *Sal* I (exon 3), or *Bam*HI (exon 4), blunted, and ligated with either *Hin*dIII, *Sma*I, or *Nde*I linkers. Three WAP linker alleles were generated: the KH allele contained one *Hin*dIII linker in the *Kpn*I restriction site; the SSM allele carried one *Sma*I linker in the *Sal*I restriction site; and the BN allele had five *Nde*I linkers inserted into the *Bam*H I restriction site (Fig. 1).

Templates used to generate synthetic RNA transcripts containing both the linker-allele and the corresponding wild-type sequences were constructed by subcloning an exon containing the linker and the equivalent wild-type sequence oligonucleotide into the plasmid pBS. Plasmid pKpH contains the first exon of the KH WAP allele and a copy of the corresponding wild-type sequence; plasmid pSlM contains wild-type and allelic sequences spanning the *Sal*I site; and plasmid pBmD contains the wild-type and allelic sequences surrounding the *Bam*HI site from the fourth exon. *In vitro* transcription with either T3 or T7 RNA polymerase generated sense transcripts from linearised plasmids.

Generation of Transgenic Mice—WAP alleles were separated from vector sequences by digestion with *Eco*RI, followed by electrophoresis in an agarose gel (FMC). DNA fragments were then isolated by electroelution and purification on a ion-exchange column (NACS prep; Bethesda Research Laboratories). The fragments were microinjected into the pronuclei of zygotes obtained from C57BL6/SJL F1 female mice. Two classes of mice were produced, KH mice were generated by injection of the KH allele alone, and KSB mice were produced using an approximately equimolar mixture of the three alleles.

DNA Analysis—Transgenic founder mice were identified by Southern blot analysis of tail DNA. DNA was prepared from tail tissue by digestion with proteinase K and precipitation with ethanol. The phenol/ chloroform extractions were eliminated without adversely affecting the results of Southern or PCR analysis. Transgenic offspring were produced by breeding the founders with C57BL6/SJL F1 mice. Once a stable line was established, offspring were screened by PCR. Mice carrying the KH allele were identified by amplification of the first WAP exon and testing the product for the presence of a *Hin*dIII restriction site. Amplification of the fourth WAP exon, which in the case of the BN gene contained an additional 50 base pairs and produced a larger PCR product, was used to identify mice carrying the BN allele.

The copy number of the alleles carried by each line was calculated by quantitative PCR. Pairs of primers flanking the *Kpn*I, *Sal*I, and *Bam*HI restriction sites of the WAP gene were used to amplify the first, third, and fourth exons, respectively, from approximately 100 ng of genomic DNA. The products were denatured with sodium hydroxide dotted onto GeneScreen *Plus* and hybridized with single-stranded oligonucleotides specific for either the allelic or corresponding wild-type sequence. The hybridizations were carried out as described in the section "RNA Analysis." To account for the different hybridization efficiency and labeling of each oligonucleotide probe, blots also contained a serial dilution of the plasmids pKpH, pSIM, and pBmD. The dots were excised, and the bound radioactivity was quantitated by scintillation counting. Results were normalized against the standard serial dilution of plasmids, and the ratio of allele to wild-type sequences was calculated. The copy number of each allele in a transgenic line was determined, based on these ratios and the results of Southern blots.

Mammary Explant Culture—Mammary organ explants were prepared and cultured as described previously (5).Concentrations of hormones in organ culture were 100 ng/ml of insulin and hydrocortisone and 1 μg/ml of prolactin.

RNA Analysis—RNA was prepared from fresh mammary tissue or organ explants using guanidine thiocyanate and acid phenol (15), fractionated on a formaldehyde agarose gel, and blotted onto GeneScreen *Plus* essentially as described (16). Polyadenylated RNA was prepared by a batch method (17). Expression of the WAP gene alleles was analyzed with the aid of the following allele-specific oligonucleotide probes.

KH: 5′ GGCAACGCATGCAAGCTTGCGGTGTCAGGCA 3′

SSM: 5′ GACACAGTCGACCCCGGGGTCGACGTTGCAG 3′

BND: 5′ GTTCTCTCTGGATCCCATATGGCCATATGGC 3′

Oligonucleotides which detected the equivalent sites in the wild-type mRNA were as follows.

KPN: 5′ CAACGCATGGTACCGGTGTCA 3′

SAL: 5′ TGACACAGTCGACGTTGCAGC 3′

BAM: 5′ TTCTCTCTGGATCCAGGAGTG 3′

Hybridizations were performed in 0.4 M NaCl, 1% sodium dodecyl sulfate, 100 mg/ml denatured herring sperm DNA and contained ^{32}P-labeled oligonucleotide probes at 1 ng/ml. All oligonucleotide probes were end-labeled with [γ-^{32}P]ATP] as described previously (17). Hybridizations with the allele or wild-type-specific oligonucleotide probes were performed at 65 and 55 °C, respectively. The radioactivity associated with a band on the filter was quantitated by scintillation counting. In order to measure the relative levels of transgene expression, blots included a serial dilution of an equimolar mixture of the three synthetic transcripts generated *in vitro* from plasmids pKpH, pSlM, and pBmD. In addition a standard sample of RNA obtained from a nontransgenic lactating mouse was included on blots to demonstrate the specificity of the oligonucleotides. Based on the serial dilution of synthetic RNAs, signals obtained with the different allele-specific oligonucleotides were normalized and expression levels of alleles could be compared against each other or against the endogenous gene when applicable.

The mouse β-casein probe was a 67-mer oligonucleotide specific for the leader peptide. The mouse keratin 18 probe was generated by random priming of a cDNA insert prepared from plasmid pUC97B (18), that had been kindly provided by Dr. Robert Oshima.

RESULTS

Generation of Mice Carrying Mouse WAP Transgenes—To distinguish between the endogenous and transgenic mouse WAP mRNAs, we tagged the transgenes by inserting linkers into either exons 1, 3, or 4 of the mouse WAP gene. Three linker alleles were produced: the KH allele contained a *Hin*dIII linker in the *Kpn*I site; the SSM allele had a *Sma*I linker at the *Sal*I site; and the BN allele carried five *Nde*I linkers at the *Bam*H I site (Fig. 1). The 0.65-kb WAP mRNA transcribed from the different WAP gene alleles was detected by hybridization with antisense oligonucleotides specific for the linker insertions.

Two classes of transgenic mice were produced; KH mice, which carried KH alleles, and KSB mice, which contained all three alleles. Transgenic mice containing the three alleles were generated to evaluate whether insertion of any of the linker molecules would interfere with gene expression. From five KH and fifteen KSB founder mice, thirteen lines of mice transmitted the transgenes to their offspring. Of these founder mice, three KH (3350A, 3350B, 3350C) and four KSB lines

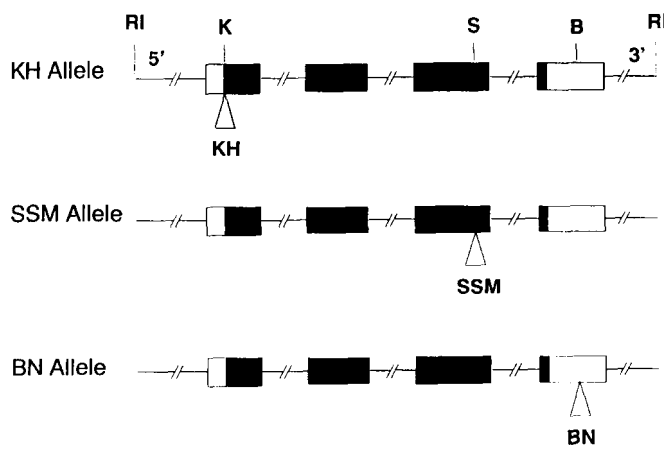

FIG. 1. **Structure of the WAP gene alleles.** A 7.2-kb genomic fragment spanning the WAP gene was modified by the insertion of oligonucleotide linkers into either the *Kpn*I (*K*), *Sal*I (*S*), or *Bam*HI (*B*) restriction sites, producing three different alleles, KH, SSM, and BN, respectively. The *open* and *closed boxes* represent untranslated and translated regions of the four WAP gene exons, respectively.

(3336, 3441, 3628, 3631) expressed the WAP transgenes during lactation at levels between 3 and 54% of the endogenous WAP gene. Expression was confined to the mammary gland (data not shown). A comparison of WAP transgene copy number and expression between six of these lines indicated that the transgene was expressed in a position dependent and copy number independent manner (Table I). Apparently the 7.2-kb WAP transgene does not represent a complete regulatory unit. However, expression levels of this construct were at least an order of magnitude higher than of hybrid genes containing just the WAP gene promoter (4, 13), suggesting the presence of additional regulatory elements downstream of the promoter.

Since the three WAP gene alleles cointegrated in KSB mice in a tandem head-to-tail arrangement (data not shown), we were able to investigate whether expression of the transgenes, which was sensitive to the surrounding chromatin (Table I), was also subject to position effects within the transgene locus. The copy number for each transgenic allele within the loci of the KSB lines was determined by quantitative PCR and was correlated with the steady state levels of the different RNA transcripts (Table II). In the three lines tested, expression of the WAP SSM and BN transgene alleles was proportional to their copy number within the transgene array, suggesting that alleles within a locus are equally active. However, the two copies of KH alleles of line 3336 were only expressed at 50% of the other alleles, and the single copy of KH allele in line 3441, situated at the border of the transgene cluster as judged by Southern analysis (data not shown), was also expressed at significantly lower levels than the SSM and BN alleles. Taken together the results suggest that most of the WAP transgenes within a transgene locus were expressed and that the level of expression among them was similar. Nevertheless, consistently lower expression of KH transcripts in different lines may indicate that the *Hin*dIII linker did interfere quantitatively with expression of the transgene.

Regulation of WAP Transgene Alleles during Development—RNA prepared from the mammary glands of female transgenic mice at various stages of development, from virgin through pregnancy and lactation, was analyzed on replicate Northern blots for either the endogenous WAP mRNA or transcripts from the different WAP transgene alleles. The endogenous gene was induced between 13 and 17 days of pregnancy and expression peaked around mid-lactation (Figs. 2 and 3). However, the expression of WAP transgenes differed qualitatively and quantitatively from the endogenous gene (Fig. 2). The developmental patterns obtained with lines 3350B and 3336 illustrate two general aspects of WAP transgene expression. First, although the absolute levels of transgene expression differed between lines of mice, the patterns of induction during pregnancy were similar. Transgenic WAP mRNA was detected at day 13 of pregnancy, preceding induction of the

TABLE I

Comparison between WAP transgene copy number and transgene expression in six independent lines of mice

Transgene copy number (per diploid genome) was determined by quantitative PCR. Transgene expression was quantitated by Northern analysis of total RNA prepared from mice at day 2 of lactation hybridized with oligonucleotides specific for either the endogenous or transgenic WAP gene (WT).

Line	Copy No.	Expression of WT
		%
3350 A	11	45
3350 B	15	54
3350 C	7	3
3336	12	46
3628	20	34
3441	14	18

TABLE II

Expression of different WAP transgene alleles within a single locus

Expression of WAP alleles in mice of three KSB lines at day 17 of pregnancy (P17) and day 2 of lactation (L2) were quantitated by Northern blots and divided by the number of copies of the allele (per cell) present in the line.

Line	Stage	Allele		
		KH	SSM	BND
		cpm/copy		
		2[a]	8	2
3336	P17	84	184	154
	L2	188	349	308
		1	5	8
3441	P17	42	172	124
	L2	30	246	256
	L2	ND[b]	88	93
		1	10	9
3628	P17	ND	86	99
	L2	ND	313	332
	L2	ND	534	510

[a] Indicates copy number.
[b] ND, not determined.

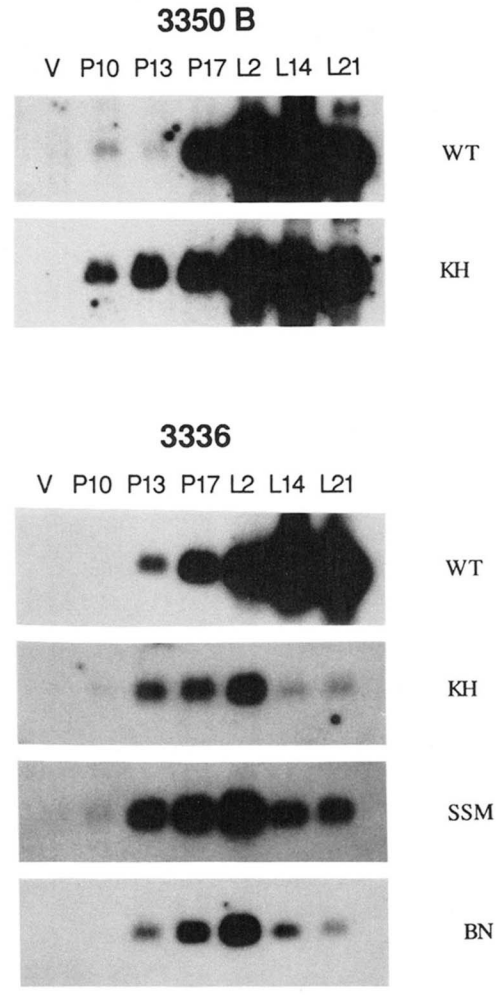

FIG. 2. **Analysis of endogenous and transgenic WAP RNA during mammary development.** Ten μg of total RNA prepared from the mammary glands of virgin mice (*V*) and at various days during pregnancy (*P*) and lactation (*L*) were analyzed on replicate Northern blots. Filters were hybridized with oligonucleotides specific for either the transgenic or wild-type (*WT*) WAP mRNAs.

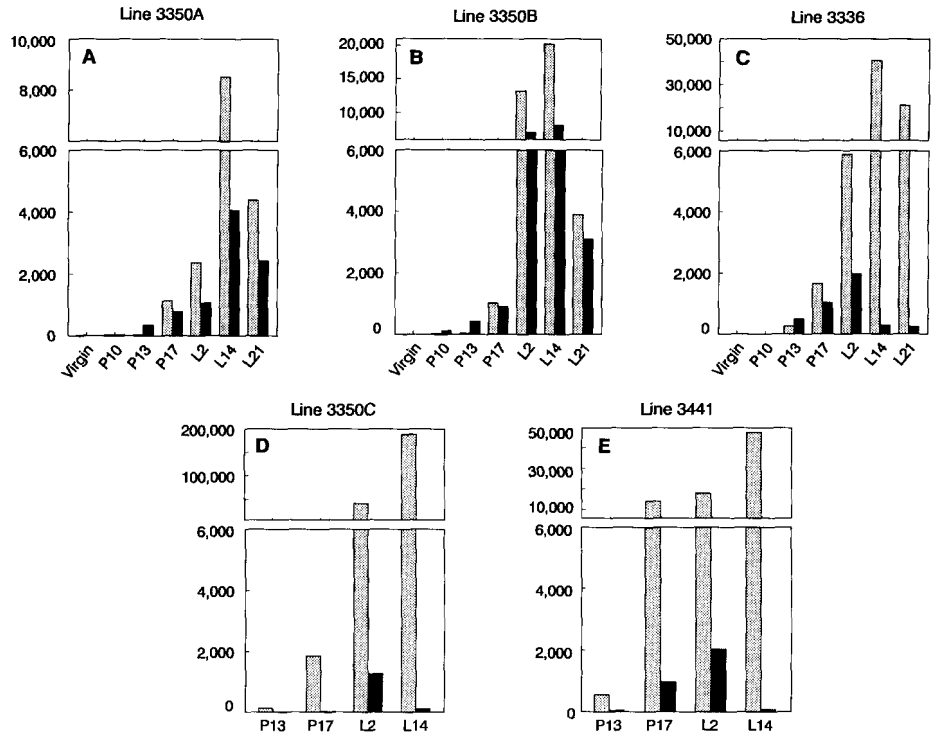

FIG. 3. **Developmental patterns of WAP transgene expression in five lines of mice.** Levels of endogenous (*dotted bars*) and transgenic (*solid bars*) WAP RNAs during mammary development were quantitated by scintillation counting the respective bands from Northern blots (see Fig. 2). RNA samples from lines 3350 C and 3441 contained poly(A$^+$)-enriched RNA equivalent to 100 μg of total RNA. A direct comparison between the level of endogenous and allele-specific WAP mRNAs within one line was made possible by correcting for the different hybridization efficiencies of oligonucleotides using synthetic RNA standards (see "Materials and Methods"). *A*, line 3350A; *B*, line 3350B; *C*, line 3336; *D*, line 3350C; *E*, line 3441. Counts/min are shown on the *ordinate*, and the developmental time points are shown on the *abssissa*. *P* refers to the respective number of days of pregnancy and *L* to the number of days into the lactational period.

TABLE III

Expression of the WAP transgenes relative to the endogenous WAP gene during mammary development

The level of expression of the WAP transgenes at various stages during mammary gland development was quantitated on Northern blots and is presented as a percentage of the endogenous WAP gene at that stage.

Line	Allele	Expression of allele as percentage of endogenous WAP				
		P13	P17	L2	L14	L21
3350 A	KH	850	70	45	48	55
3350 B	KH	1080	90	54	40	80
3336	SSM	180	63	34	1	1
3441	BN	24	7	11	0.2	ND[a]
3350 C	KH	17	2	3	0.1	ND

[a] ND, not determined.

endogenous gene by at least 2 days. Furthermore, whereas the endogenous gene is induced about 1000-fold between day 13 of pregnancy and parturition, expression of the transgenes during this period in all lines, except 3350 C, increased less than 10-fold. Second, the activities of the WAP transgenes during lactation differed dramatically between lines of mice. Expression either continued to increase approximately to the same extent as the endogenous gene, as seen in lines 3350 A and B, or expression declined as in lines 3336, 3441 and 3350 C (Figs. 2 and 3). Presenting the concentration of transgenic WAP mRNA as a percentage of the endogenous mRNA further emphasizes the premature expression of transgenes, the similarities between the patterns of transgene expression during pregnancy, and differences during lactation (Table III).

The aberrant developmental expression of the WAP transgene alleles in different lines, especially during lactation, does not appear to be caused by the linker insertions or by differential expression of individual WAP genes within transgene loci. The three WAP gene alleles in the KSB lines 3336, 3441, and 3628 were integrated in a single locus, and expression during mammary development was coordinately regulated as representatively shown for line 3336 (Fig. 2).

In Vitro Hormonal Regulation of the WAP Transgenes in Mammary Tissue from Pregnant Mice—To correlate the induction of the transgenes during development to hormonal regulation, we analyzed the expression of the WAP transgenes in an organ culture system. The WAP transgenes in lines 3350B and 3336 were already active in mammary tissue from mice which were 13–15 days pregnant (Fig. 4). In the presence of insulin, hydrocortisone, and prolactin, transgene expression in tissue from mice of lines 3350B and 3336 increased 4- and 2-fold, respectively (Fig. 4). Under the same conditions the endogenous WAP gene was induced over 30-fold. Expression of the WAP transgenes in tissue from mid-pregnant animals in the presence of insulin alone was below the level of detection, but was maintained in the presence of insulin and hydrocortisone and to a lesser extent with insulin and prolactin (Fig. 4). The levels of transgene mRNA detected in explants incubated with insulin and hydrocortisone were not due to retention of pre-existing mRNA but arose from continuing transcription of the transgenes, since comparable levels endogenous WAP mRNA were not maintained under these conditions in organ explants from late pregnant and lactating animals (Fig. 5 and data not shown).

Rehybridizations of RNA from explant cultures with a probe specific for the mouse β-casein mRNA demonstrated that, in common with the WAP transgenes, significant levels of β-casein mRNA were present at day 13 of pregnancy; induction in the presence of insulin, hydrocortisone, and prolactin (*IFP*) was only about 4-fold (Fig. 4). However, whereas expression of the WAP transgene appears to be more dependent on hydrocortisone than prolactin, the reverse is true for the β-casein gene (Fig. 4). Furthermore, the similarity between the β-casein blots from different explant experiments indicated that the response of the transgenes in different lines of mice can be compared directly.

In Vitro Hormonal Regulation of the WAP Transgenes in Mammary Tissue from Lactating Mice—The decline in expression of WAP transgenes during lactation in lines 3336,

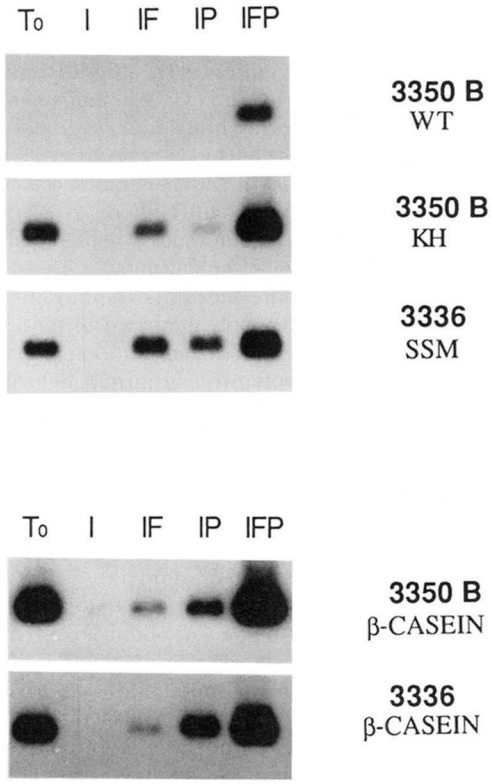

FIG. 4. **Hormonal regulation of WAP transgenes in mammary tissue from mid-pregnant mice.** Mammary explants were prepared from 13 to 15 day pregnant mice and RNA was extracted at the time of preparation (T_0) or after 48 h in culture in the presence of insulin (*I*), insulin and hydrocortisone (*IF*), insulin and prolactin (*IP*), or insulin, hydrocortisone, and prolactin (*IFP*). Northern blots carrying 5 μg of total RNA/lane were hybridized with oligonucleotides specific for either WAP alleles (*upper panel*) or mouse β-casein (*lower panel*).

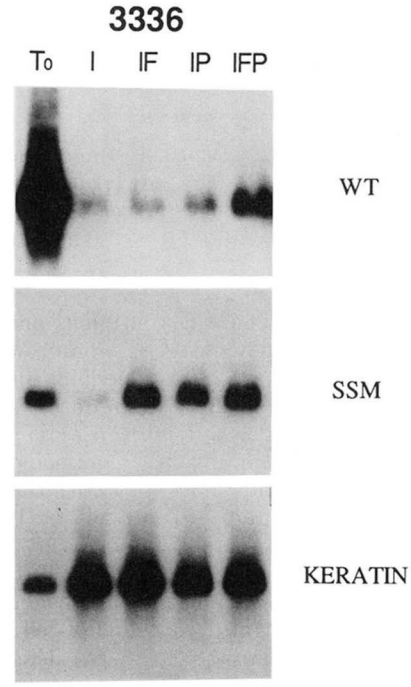

FIG. 5. **Hormone-dependent expression of WAP transgenes in mammary tissue from lactating mice of line 3336.** Mammary explants were prepared from a 10-day lactating 3336 mouse, and RNA was extracted at the time of preparation (T_0) or after 48 h in culture in different combinations of insulin (*I*), hydrocortisone (*F*), and prolactin (*P*). Northern blots carrying 5 μg of total RNA/lane were hybridized with oligonucleotides specific for either WAP alleles or a DNA probe generated from a mouse keratin 18 cDNA (12).

3441, and 3350 C raised the question whether expression of the transgenes was generally repressed or had lost its response to a particular hormone. Tissue explants prepared from a fully lactating mouse from line 3336 were cultured under the conditions employed in the induction experiments shown in Fig. 4. Although the level of endogenous WAP RNA decreased at least 100-fold under any of the hormonal conditions, levels of the transgenic mRNA either increased marginally in the presence of insulin, hydrocortisone, and prolactin (*IFP*) or remained the same with insulin and either hydrocortisone or prolactin (Fig. 5). Expression of keratin 18, a gene expressed in simple epithelial cells (18), increased during organ culture and was unaffected by hydrocortisone or prolactin. The overall response of the transgenes to hormones in tissue from lactating mice from line 3336 was similar to that obtained with tissue from pregnant mice. These results support the notion that the decrease in expression of the WAP transgenes during lactation in line 3336, and presumably also in lines 3441 and 3350 C, was due to a lack of sensitivity to the regulatory factors responsible for WAP gene induction during lactation.The transgenic WAP mRNA present during lactation in these lines probably reflects a basal level of transcription. A similar result was obtained *in vivo* with mammary tissue from mice of line 3336 where, after 3 days of weaning, levels of the endogenous WAP mRNA decreased about 100-fold and expression of transgenic WAP mRNA remained constant (data not shown).

DISCUSSION

Transgenic mice provide a unique tool for studying the developmental regulation of genes (19–21), especially those such as the milk protein genes, which are only appreciably expressed in terminally differentiated cells (5, 22–24). We introduced a virtually unaltered 7.2-kb fragment, including the entire transcribed region of the mouse WAP gene, into mice and studied its developmental and hormonal regulation. The level of expression of the WAP transgene was at least an order of magnitude higher than several hybrid genes containing the WAP promoter (4, 5, 13, 25, 26). However, expression during mammary development and upon hormonal stimulation *in vitro* differed from the endogenous WAP gene. By comparing the developmental patterns of expression, and hormonal regulation of the WAP transgenes in six lines of mice, novel aspects of WAP gene regulation became apparent.

In contrast to the variation in overall patterns of transgene expression during mammary development, and especially during lactation (Fig. 3), the activity of WAP transgenes during mid-pregnancy in different lines of mice was similar. In all lines examined, expression of the WAP transgenes was detected at day 13 of pregnancy, thereby preceding the induction of the endogenous gene. Premature activation during pregnancy has also been observed with a hybrid gene containing the mouse WAP gene promoter (5) and a rat WAP transgene (27). Furthermore, between day 13 of pregnancy and parturition, a period in which the endogenous WAP gene was induced almost 1000-fold, expression of WAP transgenes in all lines except one increased less than 10-fold.

Results from organ culture experiments with mammary tissue from pregnant mice provided an insight into the basis of the temporal deregulation of the WAP transgene. The premature expression of WAP transgenes during pregnancy was presumably related to the high basal activity of the transgene; the activity was maintained in explant cultures with hydrocortisone and insulin, both of which are present throughout pregnancy. These observations suggest that the

transgene lacks a repressor element, or alternatively, the transgene array or surrounding chromatin results in the functional loss of a repressor. Consistent early expression and variation in both patterns of induction during pregnancy and response to hormones *in vitro* suggest that both situations may operate. Whereas the 30-fold induction of the endogenous WAP gene *in vitro* required the synergistic action of insulin, hydrocortisone, and prolactin, expression of the transgenes was only marginally induced by prolactin. The small *in vitro* induction of the WAP transgenes by prolactin correlates with the modest increase in expression prior to parturition and is presumably a consequence of the prematurely elevated expression levels in mid-pregnancy. In a manner similar to the response of a WAP-tPA transgene (5), prolactin was not necessary to maintain the level of transgenic WAP RNA in organ culture, but was required for continued expression of the endogenous WAP gene. Taken together the results are consistent with prolactin acting on the endogenous WAP gene through the release of repression, thereby facilitating access of the gene to regulatory factors dependent on insulin and hydrocortisone. In support of this model, results obtained with a cell culture system suggest that the mouse β-casein promoter is induced by hormones through the release of transcriptional repression.[2]

In contrast to the situation in pregnancy, expression of the WAP transgenes in individual lines of mice differed dramatically during lactation. In two lines expression of the WAP transgene approximately followed the pattern of the endogenous WAP gene. However, in three other lines of mice, in which the transgenes were induced during pregnancy and responded to hormones in organ culture, expression decreased in response to lactation. To obtain the same effect in three independent lines of mice demonstrates that loss of induction was not peculiar to one site of integration, but that the transgene lacks a regulatory element necessary to ensure correct expression during lactation. Günzburg and co-workers (28) have also reported the down-regulation of a WAP-hGH transgene in transgenic mice. Although a decrease in the expression of a 4.3-kb rat WAP transgene during lactation was not reported (27), this phenomenon may not have been detected, because developmental studies were performed on only two lines of mice and at a single time point during lactation. Alternatively, the rat and mouse WAP genes may have a different arrangement of regulatory elements.

Our results indicate that high level WAP gene expression during lactation is mediated by a combination of elements or mechanisms distinct from those operating during pregnancy. This is supported by the inability to maintain high levels of endogenous WAP RNA in tissue from lactating mice, under conditions sufficient to induce expression in tissue from pregnant mice. Expression of keratin 18 mRNA in culture (Fig. 5.) demonstrated that the decrease in endogenous WAP mRNA *in vitro* was not due to a general decrease in gene expression. Appropriate induction of WAP transgenes in two lines of mice (3350 A, 3350 B) during lactation indicates that chromatin at the sites of integration may have provided a positive influence which enabled the transgene to be appropriately induced during lactation. A similar facilitating effect of flanking chromatin has been described in a deletion analysis of the locus activating region of a human adenosine deaminase transgene.[3]

The coordinate developmental regulation and equivalent expression of different WAP transgene alleles within a single locus suggest that position effects are exerted on the transgene array as a whole and are not due local effects within the transgene cluster. This supports the notion that position effects may be mediated by some general characteristic of the surrounding chromatin, probably due to regulatory elements at some distance from the transgene array (29), and are not due to a local enhancer which might be expected to polarize expression within the transgene locus. These results demonstrate the feasibility of analyzing the function of mutated genes, relative to an unaltered control gene, by cointegration of transgenes in transgenic mice.

Acknowledgments—We thank the members of the Laboratory of Biochemistry and Metabolism for their advice, Robert Oshima for a mouse keratin cDNA clone, and W. Jakoby for his most generous support.

REFERENCES

1. Hobbs, A. A., Richards, D. A., Kessler, D. J., and Rosen, J. M. (1982) *J. Biol. Chem.* **257,** 3598–3605
2. Hennighausen, L. G., and Sippel, A. E. (1982) *Eur. J. Biochem.* **125,** 131–141
3. Devinoy, E., Hubert, C, Jolivet, G., Thepot, D., Clergue, N., Desaleux, M., Servely, J.-L., and Houdebine, L.-M. (1988) *Reprod. Nutr. Dev.* **28,** 1145–1164
4. Pittius, C. W., Sankaran, L., Hennighausen, L., Lee, L., Westphal, H., Nichols, E., Vitale, J., and Gordon, K. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 5874–5878
5. Pittius, C. W., Lackshmanan, S., Topper, Y. J., and Hennighausen, L. (1988) *Mol. Endocrinol.* **2,** 1027–1032
6. Chen, L., and Bissell, M. J. (1990) *Cell Regul.* **1,** 45–54
7. Thoradson, G., and Talamantes, F. (1984) in *The Mammary Gland: Development, Regulation and Function* (Neville, M. C., and Daniel, C. W., eds) pp. 459–498, Plenum Press, New York
8. Hennighausen, L., Westphal, C., Sankaran, L., and Pittius, C. W. (1990) in *Transgenic Animals* (First, N., and Haseltine, F. P., eds) pp. 65–74, Butterworth-Heinemann, Stoneham, MA
9. Lee, K.-F., Atiee, S. H., Henning, S. J., and Rosen, J. M. (1989) *Mol. Endocrinol.* **3,** 447–453
10. Schoenenberger, C.-A., Zuk, A., Groner, B., Jones, W., and Andres, A.-C. (1990) *Dev. Biol.* **139,** 327–337
11. Doppler, W., Groner, B., and Ball, R. K. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 104–108
12. Eisenstein, R. S., and Rosen, J. M. (1988) *Mol. Cell. Biol.* **8,** 3183–3190
13. Andres, A., Schoenenberger, C., Groner, B., Hennighausen, H., LeMeur, M., and Gerlinger, P. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 1299–1303
14. Campbell, S. M., Rosen, J. M., Hennighausen, L. G., Strech-Jurk, U., and Sippel, A. (1984) *Nucleic Acids Res.* **12,** 8685–8697
15. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
16. Kroczek, R. A., and Seibert, E. (1990) *Anal. Biochem.* **184,** 90–95
17. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Singer, P. A., Trevor, K., and Oshima, R. (1986) *J. Biol. Chem.* **261,** 538–547
19. Behringer, R. R., Ryan, T. M., Palmiter, R. D., Brinster, R. L., and Townes, T. M. (1990) *Genes & Dev.* **4,** 380–389
20. Idzerda, R. L., Behringer, R. R., Theisen, M., Huggenvik, J. I., McKnight, G. S., and Brinster, R. L. (1989) *Mol. Cell. Biol.* **9,** 5154–5162
21. Hammer, R. E., Swift, G. H., Ornitz, D. M., Quaife, C. J., Palmiter, R. D., Brinster, R. L., and MacDonald, R. J. (1987) *Mol. Cell. Biol.* **7,** 2956–2967
22. Simons, J. P., McClenaghan, M., and Clark, A. J. (1987) *Nature* **328,** 530–532
23. Lee, K.-F., Demayo, F. J., Aitee, S. H., and Rosen, J. M. (1988) *Nucleic Acids Res.* **16,** 1027–1041
24. Vilotte, J. L., Soulier, S., Stinnakre,M.-G., Massoud, M., and Mercier, J.-C. (1989) *Eur. J. Biochem.* **186,** 43–48
25. Schoenenberger, C., Andres, A., Groner, B., van der Valk, M., LeMeur, M., and Gerlinger, P. (1988) *EMBO J.* **7,** 169–175
26. Tomasetto, C., Wolf, C., Rio, M.-C., LeMeur, M., Gerlinger, P., Chambon, P., and Lathe, R. (1989) *Mol. Endocrinol.* **3,** 1579–1584
27. Bayna, E. M., and Rosen, J. M. (1990) *Nucleic Acids Res.* **18,** 2977–2985
28. Günzburg, W. H., Salmons, B., Zimmermann, B., Müller, M., Erfle, V., and Brem, G. (1991) *Mol. Endocrinol.*, in press
29. Al-Shawi, R., Kinnaird, J., Burke, J., and Bishop, J. O. (1990) *Mol. Cell. Biol.* **10,** 1192–1198

[2] W. Doppler, personal communication.

[3] B. Aronow, personal communication.